Review Article

Analytical methods for determination of Amphotericin B in biological samples: a short review

Gurpreet Singh*, Tanurajvir Kaur, Anudeep Kaur, Ravinder Kaur, Ramandeep Kaur

Department of Pharmaceutical Sciences Guru Nanak Dev University, Amritsar Punjab (INDIA)

Keywords: *Amphotericin B, Biological samples, Plasma, Human CSF, Piroxicam, HPLC*

Abstract

Determination of Amphotericin B in biological samples is of great importance, not only in clinical and forensic toxicology, but also in drug testing and pharmacokinetic profile. Human biological samples including organs, tissues, biofluids such as blood and their derivatives, are increasingly important resources for biomedical research. They can help us to understand how a diagnosis can be carried out, categorized and treatment can be further imitated for a whole variety of medical condition and are particularly important when studying pharmacokinetic profile of drugs in animal models. This review paper is a compilation of last 60 years research articles from 1954 to 2013 that describe procedures for the detection of Amphotericin B, in enormous variety of biological samples. The efficient analytical techniques assigned for accurately analyzing and quantifying drug in biological samples are HPLC and LC MS/MS (hyphenated techniques). Furthermore, this review provides all basic information regarding the sample preparation, type of biological sample and all detail concerning the determination through chromatography.

***Corresponding author:**
Gurpreet Singh, Assistant Professor (Pharmaceutics), Department of Pharmaceutical Sciences Guru Nanak Dev University, Amritsar Punjab (INDIA) 143005
E-mail: gurpreet_pharma85@yahoo.com; Mobile No: +91-9814085601

Date of Submission: Oct 8, 2014 **Date of Acceptance:** Oct 12, 2014 **Date of Publishing:** Nov 3, 2014

How to cite this paper:
Singh G, Kaur T, Kaur A, Kaur R. Analytical methods for determination of Amphotericin B in biological samples: a short review. Annals of Applied Bio-sciences. 2014;1:R26-32

Introduction

Amphotericin B (AmB) is a polyene antifungal antibiotic produced by *Streptomyces nodosus* and used in clinical practice since the 1960s. Two amphotericins (A and B) were isolated in the 1950s from *Streptomyces nodosus*, an aerobic bacterium, from a soil sample from Venezuela's Orinoco River Valley by Gold and coworkers in the 1950s.[1] Amphotericin B having molecular formula of $C_{47}H_{73}NO_{17}$ and chemical structure as shown in Figure 1. It has seven conjugated double bonds, an internal ester, a free carboxyl group, and aglycoside side chain with a primary amino group. AmB bind to ergosterol in the fungal membrane forming a channel in the membrane with the hydrophilic surface facing the interior of the channel. This permits leakage of intracellular components (K^+, Na^+, H^+ and Cl^-), leading to fungal death.[2]

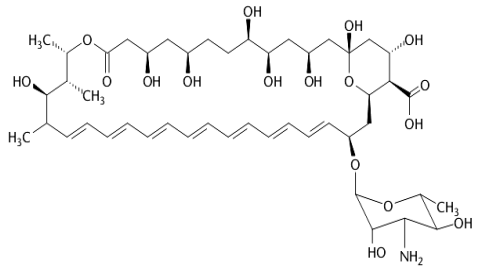

Figure 1: Chemical structure of Amphotericin B

AmB is poorly soluble in water and has limited oral absorption. Limited use of AmB is confined due to its dose dependent nephrotoxicity which is frequent and severe. But still remains the most effective drug in treatment of systemic fungal infections and regained wide clinical applications.[3-5] In recent years, new formulations such as liposomal amphotericin B have been developed that have significantly minimized nephrotoxicity and side effects. Currently, several lipid-based formulations of AmB have been developed such as a liposomal formulation (AmBisome®), a lipid complex of AmB with phospholipids (ABELCET®) and a colloidal dispersion of AmB with cholestryl sulfate (Amphocil®) and they are now commercially available. These formulations have been developed to improve tolerability for the patient, but may show considerably different pharmacokinetic characteristics as compared conventional AmB.[6] However, developing a safe and effective drug/drug delivery system is not only part of the picture; its determination in biological sample as well as in pharmaceutical dosages form with aid of most accurate method plays a significant role in formulation development with clinical relevance. Physicochemical and pharmacokinetic properties of a drug are another point of consideration for selection of suitable method for its determination in sample. Physicochemical and pharmacokinetic properties of AmB are summarized in Table 1 and Table 2.[3, 7-10]

Table 1: Physicochemical properties of Amphotericin B

Category	Polyene Antibiotic	
CAS Number	1397-89-3	
ATC Number	A01AB04; A07AA07; G01AA03; J02AA01.	
Empirical Formula	$C_{47}H_{73}NO_{17}$	
Molecular weight	924.08	
IUPAC Name	(1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-{[(2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyloxan-2-yl]oxy}-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriacontata-19,21,23,25,27,29,31-heptaene-36-carboxylic acid.	
Solubility	N,N-dimethylformamide N,N-dimethylformamide + hydrochloric acid Dimethyl sulfoxide Water at pH 2 or pH 11	2-4 mg/mL 60-80 mg/mL 30-40 mg/mL 0.1 mg/mL
Melting Point:	170°C	
pKa	5.7 (carboxyl) and 10.0 (amine)	
logP	0.8	
IR Bands	1010, 1065, 1038, 1103, 1183, 1126 cm^{-1} (KBr disk)	
UV λ max	406, 382, 363 and 345 nm	

Table 2: Pharmacokinetics of Amphotericin B

Parameter	Value
Absorption	Oral <5%, Intramuscular poor
Half-life	Initial phase 24-48 h, Terminal phase 15 d
Apparent volume of distribution	Overall 4 L/kg, Central compartment 0.44 L/kg Fast compartment 0.35 L/kg, Slow compartment 3.20 L/kg
Urinary recovery at 24 h	3%
Binding to β-lipoproteins	91-95%

Determination of Amphotericin B in biological samples

Several assay methods are reported in the literature for the determination of AmB in biological samples while accounted with their individual limitations. Microbiological techniques have been used extensively for the determination of AmB in serum.[11-13]

But not have been widely followed confining to relatively less précised**,** difficult to standardize and slow in comparison to instrumental analytical methods and when there is a need for rapid clinical feedback of the AmB levels. Other method which can be opted is spectrophotometric assay [14-17] but there are chances of interferences due to the presence of heme and other colored substances in estimation of AmB. The most widely used methods include HPLC methods which are used for determination of AmB in biological fluids. In some methods internal standard are not employed, which

requires careful sample preparation in order to reduce the chances of errors.[18-22] while other methods has internal standards as one of the component which increases the running cost of assay[23-35] such as Piroxicam, Natamycin, p-nitroaniline etc. Problem associated with HPLC methods are large volume of plasma is needed for studies which is difficult and comes restriction to quantification of AmB in plasma or serum and are not ideal for other biological samples because of higher limits of quantification and long chromatographic run times. Advanced hyphenated technique such as LC-MS/MS is an alternative to HPLC methods for determination of AmB in other biological samples generally which has better selectivity and sensitivity than HPLC methods.[36-38] The data for determination of AmB in biological samples for pharmacokinetics as well as for toxicological studies have been appended in proceeding pages in Table 3 with important process variables which impact on rapidity, accuracy and limit of detection (LOD) for quantification of drug.

Conclusion

Bio-analytical methods are the most powerful analytical tool for screening and identification of AmB in biological matrices as compared to microbiological methods. They are more sensitive, have low detection limit, accurate, précised and high recovery. However, adequate sample preparation is a key prerequisite aspect of successful quantitative and qualitative analysis. Among all of the published methods, the most frequently used method for quantification of AmB is HPLC coupled to mass spectrometer or UV detector. The LC-MS/MS methods are considered as most appropriate method for quantification of AmB in other biological samples. The ultimate goal is to obtain results with more and more precision and accuracy and at increasingly lower concentration levels of the AmB being determined in biological samples. Its a continues process, with advancement of technology, new analytical methods could be developed in future, but in current status HPLC methods are most commonly used for plasma and serum samples with exception to low concentration of AmB in certain biological samples/fluids.

Acknowledgements

None.

Funding

None.

Competing Interests

None declared.

References

1. Lederberg J. Antifungal agents. In: Schaechter M, editor. The Desk Encyclopedia of microbiology, 1st ed. UK: Academic Press; 2004.p. 47-67.
2. Hartsel S, Bolard J. Amphotericin B: new life for an old drug. Trends Pharmacol Sci. 1996;17:445-9.
3. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis. 1990;12:308-29.
4. Hann IM, Prentice HG. Lipid-based amphotericin B: a review of the last 10 years of use. Int J Antimicrob Agents. 2001;17:161-9.
5. Hamill RJ. Amphotericin B formulations: a comparative review of efficacy and toxicity. Drugs. 2013;73:919-34.
6. Cifani C, Costantino S, Massi M, Berrino L. Commercially available lipid formulations of amphotericin B: are they bioequivalent and therapeutically equivalent? Acta Biomed. 2012;83:154-63.
7. Amphotericin B. In: Florey K, editor. Analytical Profiles of Drug Substances, vol 6. UK: Academic Press; 1977. p.1-42.
8. Amphotericin B. In: Moffat AC, editor. Clarkes's Isolation and Identification of Drugs, 4th ed. Pharmaceutical Press; 2011, p.897.
9. Antifungals. In: Sweetman SC, Martindale: The Complete Drug Reference, 36th ed. Pharmaceutical Press; 2009, p.523-7.
10. Amphotericin B. http://www.drugbank.ca/drugs/DB00681 accessed on 1/3/2013
11. Tarbet JE, Sternberg TH. Microbiological estimation of antifungal blood levels following administration of fungicides. Mycologia. 1954;46:263–75.
12. Halde C, Newcomer VD, Wright ET, Sternberg TH. An evaluation of amphoteri-

cin B *in vitro* and *in vivo* in mice against Coccidioides immitis and Candida albicans, and preliminary observations concerning the administration of amphotericin B to man. J Invest Dermatol. 1957;28:217-32.
13. Shadomy S, McCay JA, Schwartz SI. Bioassay for hamycin and amphotericin B in serum and other biological fluids. Appl Microbiol. 1969;17:497-503.
14. Herd PA, Martin HF. A semi-quantitative spectrophotometric assay for amphotericin B in blood. Clin Biochem. 1974;7:359-65.
15. Millership JS, McCaffrey F, Tierney D. A novel application of ratio spectra derivative spectroscopy for the determination of amphotericin in highly icteric plasma. J Pharm Biomed Anal. 2008;48:408-13.
16. Ganière MC, Kergueris MF, Iooss P, Thomas L, Larousse C. Quantitation of amphotericin B in plasma by second-derivative spectrophotometry. J Pharm Biomed Anal. 1998;17:481-5.
17. Botsoglou NA, Fletouris DJ, Papageorgiou GE, Florou-Paner P, Mantis AJ. Rapid determination of amphotericin B in serum and urine by third-order derivative spectrophotometry. J Pharm Sci. 1996;85:402-6.
18. Pavan B, Dileep RJ, Nivesh KM, Bivash M, Laura AT, George CW. Rapid Quantitative Evaluation of Amphotericin B in Human Plasma, by Validated HPLC Method. Journal of Bioequivalence & Bioavailability. 2013;5:121-4.
19. Espada R, Josa JM, Valdespina S, Dea MA, Ballesteros MP, Alunda JM, Torrado JJ. HPLC assay for determination of amphotericin B in biological samples. Biomed Chromatogr. 2008;22:402-7.
20. Nilsson EI, Yoshikawa TT, Edwards JE, Schotz MC, Guze LB. Quantitation of amphotericin B with use of high-pressure liquid chromatography. J Infect Dis. 1977;135:414-22.
21. Koizumi T, Kubo K, Kaneki T, Hanaoka M, Hayano T, Miyahara T, et al. Pharmacokinetic evaluation of amphotericin B in lung tissue: lung lymph distribution after intravenous injection and airspace distribution after aerosolization and inhalation of amphotericin B. Antimicrob Agents Chemother. 1998;42:597-600.
22. Lemke A, Kiderlen AF, Petri B, Kayser O. Delivery of amphotericin B nanosuspensions to the brain and determination of activity against Balamuthia mandrillaris amebas. Nanomedicine. 2010;6:597-603.
23. Yang L, Xiao P, Shi C, You J, Dong X, Zhang Y. Comparative pharmacokinetics after administration of a novel formulation of amphotericin B nanoparticles and amphotericin B deoxycholate to rats and their antifungal activity in vitro. Asian J Pharm Sciences. 2007;2:122-9.
24. Bach PR. Quantitative extraction of amphotericin B from serum and its determination by high-pressure liquid chromatography. Antimicrob Agents Chemother. 1984;26:314-7.
25. Hosotsubo H, Hosotsubo K. Improved high-performance liquid chromatographic determination of amphotericin B in human serum and plasma. J Pharm Biomed Anal. 1989;7:975-9.
26. Liu H, Davoudi H, Last T. Determination of amphotericin B in cerebrospinal fluid by solid-phase extraction and liquid chromatography. J Pharm Biomed Anal. 1995;13:395-400.
27. Echevarría I, Barturen C, Renedo MJ, Dios-Viéitez MC. High-performance liquid chromatographic determination of amphotericin B in plasma and tissue. Application to pharmacokinetic and tissue distribution studies in rats. Journal of Chromatography A. 1998; 819:171–6.
28. Zou SR, Li YQ, Li P, Zhou B, Wen YG. Determination of amphotericin B in human plasma by HPLC. Journal of Guangdong Pharmaceutical College. 2010;6:602-4.
29. Campanero MA, Zamarreno AM, Diaz M, .Dios-Vieitez MC, Azanza JR. Development and validation of an HPLC method for determination of amphotericin B in plasma and sputum involving solid phase extraction. Chromatographia. 1997;46:641-6.
30. Italia JL, Singh D, Ravi Kumar MN. High-performance liquid chromatographic analysis of amphotericin B in rat plasma using α-naphthol as an internal standard. Anal Chim Acta. 2009;634:110-4.
31. Egger P, Bellmann R, Wiedermann CJ. Determination of amphotericin B, liposomal amphotericin B, and amphotericin B colloidal dispersion in plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2001;760:307-13.
32. Uday SC, Tapan KP. Rapid and Sensitive High Performance Liquid Chromatography Method for the Determination of Amphotericin B in Rat Plasma. J Pharm Research. 2011;4:3194-7.

33. Goldblum D, Rohrer K, Frueh BE, Theurillat R, Thormann W, Zimmerli S. Ocular distribution of intravenously administered lipid formulations of amphotericin B in a rabbit model. Antimicrob Agents Chemother. 2002;46:3719-23.
34. Malone ME, Corrigan OI, Kavanagh PV, Gowing C, Donnelly M, D'Arcy DM. Pharmacokinetics of amphotericin B lipid complex in critically ill patients undergoing continuous venovenous haemodiafiltration. Int J Antimicrob Agents. 2013;42:335-42.
35. Alak A, Moy S, Bekersky I. A high-performance liquid chromatographic assay for the determination of amphotericin B serum concentrations after the administration of Ambisome, a liposomal amphotericin B formulation. Ther Drug Monit. 1996;18:604-9.
36. Bhatta RS, Chetan R, Chandasana H, Kumar D, Chhonker SY, Jain GK. LC–MS Method for Determination of Amphotericin B in Rabbit Tears and Its Application to Ocular Pharmacokinetic Study. Chromatographia. 2011;73:487-93.
37. Xiong X, Zhai S, Liu F. Determination of amphotericin B in human cerebrospinal fluid by. LC-MS-MS. Chromatographia. 2009;70:329-32.
38. Lee JW, Petersen ME, Lin P, Dressler D, Bekersky I. Quantitation of free and total amphotericin B in human biologic matrices by a liquid chromatography tandem mass spectrometric method. Ther Drug Monit 2001;23:268-76.

Table: 3 Analytical methods of determination of Amphotericin B in biological samples

S. No	Method	Biological sample	Internal Standard (IS)	Experimental condition	LOD	Ref.
1	Spectrotrophotomeric Method	Serum, urine	-	**System:** Shimadzu UV-160A **Sample preparation:** solvent acetonitrile added to one mL of serum or urine sample, then centrifuged and UV scan with blank water. **Detection:** 402 nm	0.2 to 2.4 µg/mL	17
2	HPLC	Human plasma	-	**System:** Waters HPLC Alliance system on an e2695 separations module with a Waters 2998 photo-diode array (PDA) detector. **Column:** Waters XBridge^{TM} C_{18} reversed-phase column (150 x 4.6 mm, 3.5 µm) **Mobile phase:** acetic acid (0.73%): acetonitrile (60:40 v/v). **Flow rate:** 1.0 mL/min **Injection volume:** 20 µl **Detection:** 408 nm	9.98 ng/mL	18
3	HPLC	Human plasma, Mice liver, spleen and kidneys	-	**System:** Jasco PU-1580 pump, a Gilson 231 XL auto-sampler fitted to a 100 µL sampling loop and a Jasco UV-1575 UV–visible detector. **Column:** Thermo Hypersil BDS C_{18} reverse-phase column (250 × 4.6 mm, 5µm) **Mobile phase:** acetonitrile : acetic acid: water (52:4.3:43.7 v/v/v) **Flow rate:**1 mL/min **Detection:** 406 nm	0.016 µg/mL	19
4	HPLC	Human, dog and rabbit Serum and cerebrospinal fluid	-	**System:** Liquid chromatograph (model no. ALC/GPC 200; Waters Associates) equipped with a model 6000 solvent delivery system, a model U6K Universal injector, a model 440 absorbance detector. **Column:** MicroBondapak C_{18} column (300 x 4 mm) **Mobile phase:** Methanol was mixed with the EDTA solution (8:2 v/v) **Injection volume:** 300 µL **Flow rate:** 2.5 mL/min **Detection:** 405 nm	0.02 µg/mL	20
5	HPLC	Sheep (Plasma, lung lymph fistulas and tracheotomy)	-	**System:** Modular HPLC (L-4250, L-6200, AS-4000; Hitachi) **Column:** Lichrospher RP-18(e) column (250 x 4mm) **Mobile phase:** 10 mM EDTA.2K-methanol (25:75 v/v) **Detection:** fluorescence with excitation and emission set at 405 nm	10 to 5,000 ng/mL.	21
6	HPLC	Brains, livers, and kidneys of mice	-	**System:** Kontron 560 HPLC system consisting of a Kontron 525 pump, Kontron UV detector 430, Auto-sampler T360, and a Kontron column heater **Column:** Nucleosil 120 C_{18} (125 × 4.6 mm, 3 µm) **Mobile phase:** eluent A (methanol, acetonitrile, and 0.00125 M EDTA (500:350:300) and eluent B (tetrahydrofuran 100%). **Injection volume:** 20 µL **Flow rate:**1.2 mL/min **Detection:** 430 nm	840 ng/mL	22
7	HPLC	Rat	Piroxicam	**Column**: Reversed-phase ODS column HiQsil. C_{18} (150 x 4.6 mm,	5 ng/mL	23

		(Plasma, Kidney, Liver, Spleen, Lung Heart)		5 µm) **Mobile phase:** acetonitrile : 0.02 M EDTA disodium salt at pH 5.0 (40:60 v/v) **Injection volume:** 10 µl **Flow rate:** 1.0 mL/min **Detection:** 406 nm		
8	HPLC	Human serum	Disperse yellow 42	**System:** Perkin-Elmer Series 2/2 pump LC-65T Detector-Oven and with a Chromatopac C-R1A data processor **Column:** Ultrasphere ODS model 256-06 (150 x 4.6 mm, 5 µm) **Mobile phase:** acetonitrile: methanol: 5mM EDTA disodium salt (187:449:354 v/v) **Injection volume:** 40 µl **Flow rate:** 1.5 mL/min. **Detection:** 386 nm	0.005µg/mL	24
9	HPLC	Human serum and plasma	p-nitroanilin e	**System:** Model LC-6A solvent delivery system, Model SIL-1A injector, and Model SPD-6AV UV detector **Column:** Reversed-phase CLC-C_8 column (150 x 6.0 mm, 5 µm) **Mobile phase:** 0.01 M sodium acetate buffer, pH 7.4: acetonitrile (60:40 v/v) **Injection volume:** 50 µl **Flow rate:** 1.2 mL/min **Detection:** 405 nm	0.01 mg/l	25
10	LC	Dog and human cerebrospinal fluid	Nystatin	**System:** Hewlett-Packard 1050 system with a Chem Station data system (version B.02.04) **Column:** Nova-Pak C_{18} (150 × 3.9 mm, 4 µm) **Mobile phase:** Mixture of 0.01M EDTA (pH 5): acetonitrile (65:35 v/v) **Injection volume:** 100 µl **Flow rate:** 0.5 mL/min **Detection:** 410 nm	0.5 ng/mL	26
11	HPLC	Rat (Plasma, liver, spleen and kidney)	Piroxicam	**System:** Waters 600E pump a Waters 700 Satellite WISP auto sampler and a Waters 486 ultraviolet detector. **Column:** Ultrabase C_{18} reversed-phase column (250 x 34.6 mm, 5 mm) proceeded by a guard column (45 x 34.6 mm, 5 mm) filled with pellicular C **Mobile phase:** acetonitrile : acetic acid (10%) : water (41:43:16) **Injection volume:** 100 µl **Flow rate:** 1 mL / min. **Detection:** 405 nm	2.0 ng/mL for plasma, 0.62, 0.42 and 1.96 mg/g for liver, spleen and kidney respectively	27
12	HPLC	Human plasma	Berberine	**Column:** RP-HPLC column Agilent TC-C_{18} (150 x4.6 mm, 5 µm) **Mobile phase:** acetonitrile 0.03M: ammonium acetate (44:56 v/v pH 4.8) **Injection volume:** 15 µl **Flow rate:** 0.8 mL/min **Detection:** 410 nm	0.04 µg/mL	28
13	HPLC	Plasma and sputum	Piroxicam	**System:** Hewlett Packard, HP 1050 quaternary pump; a HP 1050 auto-sampler and a HP 1050 diode-array detector **Column:** Reversed-phase Nucleosil C_{18} column (120 x 4 mm, 5 µm) **Mobile phase:** Acetonitrile : water: acetic acid (44:51:5 v/v) **Injection volume:** 80 µL **Flow rate:** 1 mL/min **Detection:** 407 nm	5 ng/mL for plasma and 10 ng/mL for sputum	29
14	HPLC	Rat plasma	α-naphthol	**System:** Waters HPLC system (717 plus auto-sampler and 600 controller) coupled to PDA (996). **Column:** Nucleosil® 100-5 C_{18} (150 × 4.6 mm) **Mobile phase:** acetonitrile and 10 mM acetate buffer (pH 4), gradient mode **Injection volume:** 50 µL **Flow rate:** 1 mL/min **Detection:** 407 and 294 for AmB and internal standard	5 ng/mL	30
15	HPLC	Human plasma	1-amino-4-nitronaphtha-lene	**System:** HPLC system consisting of a Model LC-6A pump, a Model SPD-6AV UV visible spectrophotometric detector and a Chromatopac CR3A integrator. **Column:** Bondapak C_{18} reversed-phase column (300 x4.6 mm, 5µm) **Mobile phase:** Acetonitrile : Disodium edetate (20 mM) (45:55 v/v) at pH 5.0 **Injection volume:** 100 µL **Flow rate:** 1 mL/min **Detection:** 407 nm	0.00500 mg /mL	31

16	HPLC	Rat Plasma	Lornoxicam	**System:** HPLC Shimadzu Model, LC-20AT HPLC pump, Rheodyne injector (D-14163 Berlin), SPD–20A variable wave length UV detector and Spinchrom (version 2.4) **Column:** Hypersil ODS, C_{18}, (250 x 4.6 mm, 5 µm) **Mobile phase:** 10 mmol phosphate buffer: acetonitrile. (65:35 v/v) **Injection volume:** 50 µL **Flow rate:** 1 mL/min **Detection:** 407 nm	3 ng/mL	32
17	HPLC	Rabbit (Aqueous humor, vitreous humor, and plasma)	1,1-diphenyl-2-pikryl-hydrazyle	**System:** HPLC setup comprised a Waters model 501 pump, a Waters 717plus auto-sampler, and a Spectro flow 757 absorbance detectors. **Column:** Nova-Pack C_{18} column (150x 4.6 mm, 4 µm) using a pH 7.5 **Mobile phase:** Methanol : acetonitrile mixture (5:95 v/v) **Injection volume:** 50µL for aqueous and vitreous humor and 20µL for plasma **Flow rate:**1 mL/min **Detection:** 380 nm	0.05 µg/mL	33
18	HPLC	Human Plasma	4-nitro-1-naphthylamine	**System:** Hewlett Packard 1050 series degasser, pump, a Jones Chromatography Column Heater model 7965, a Waters 717 auto-sampler, Shimadzu SPD 10A ultraviolet detector. **Column:** Waters symmetry C_{18} column (150 x 4.6 mm, 5 µm) attached to Waters symmetry C18 guard column (20 x 3.9 mm, 5 µm) **Mobile phase:** Gradient elution of acetonitrile from 20% to 42% plus 0.1 M acetate buffer (pH 4.2) solution from 80% to 58% in 10.5 min. **Injection volume:** 50 µL **Flow rate:** 1.25 mL/min **Detection:** 405 nm	0.01µg/mL	34
19	HPLC	Human serum	3-nitrophenol	**System:** Hitachi AS4000 Intelligent AutoSampler, a Hitachi L-6200A Intelligent Pump, and an ABI Kratos Spectrof low 758A UV/VIS Detector **Column:** (250 × 4.6 cm, 5 µm) type 316 stainless steel, packed with Alltima ODS. **Mobile phase:** acetonitrile : water containing 0.05N sodium acetate, anhydrous (34:66 v/v). **Injection volume:** 20µL **Flow rate:**1 mL/min **Detection:** 406 nm	0.05 µg/mL	35
20	LC/MS/MS	Rabbit tears	Natamycin	**System:** Perkin-Elmer Series 200 HPLC system **Column**: Luna 3µ CN column (100×2 mm, 3 µm) **Mobile Phase**: Methanol: Ammonium acetate (3.5 mM, adjusted to pH 4 with acetic acid) (90:10 v/v) **Injection volume:** 15 µl **Flow rate:** 0.3 mL/min **Mass spectrometric:** API 4000 mass spectrometer, spray voltage:-4500V, Software PE SCIEX Analyst (Ver.1.4.1). Nebulizer gas, curtain gas, auxiliary gas and collision gas set at 30, 15, 25 and 3, psi; respectively.	100 ng/mL	36
21	LC/MS/MS	Human CSF	Rifaximin	**System:** Agilent 1200 system (Agilent Technologies). **Column:** Sunfire C_{18} column (50 x 2.1 mm, 5 µm) **Mobile phase:** 0.1% Formic acid: methanol (40:60 v/v) **Injection volume:** 15 µl **Flow rate:** 0.3 mL/min **MS–MS Parameter:** Gas temperature, 350°C; nebulizer gas, 30 psi; gas flow, 8 L/min; capillary voltage, 4,500 V; fragmentor 190 V for AmB and 150 V for the IS; collision energy 16 V for AmB and 20 V for internal standard.	0.5 ng/mL	37
22	LC/MS/MS	Plasma, Urine, Fecal	Natamycin	**System:** PE Sciex and API 3000 (for free amphotericin B) or API 3+ (for total amphotericin B) triple quadrupole mass spectrometer **Column:** Waters Symmetry C_{18} column (150 × 3.0 mm, 5 µm) **Mobile phase:** methanol :water: acetic acid (68.6/29.4/1.96 v/v/v) **Injection volume:** 50 µL **Flow rate:** 0.5 mL/min **Detection:** 405 nm **MS-MS Parameter:** Turbo Ionspray for interface. High-purity nitrogen as a drying gas at a flow rate of 8 L/min. The turbo probe temperature 480°C.	2 µg/mL for plasma, 0.05 µg/mL for urine, and 0.4 µg/mL for fecal homogenate	38